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Spatial regulation of coalesced protein assemblies: Lessons from yeast to diseases

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ABSTRACT. Organisms rely on correctly folded proteins to carry out essential functions. Protein quality control factors guard proteostasis and prevent protein misfolding. When quality control fails and in response to diverse stresses, many proteins start to accumulate at specific deposit sites that maintain cellular organization and protect the functionality of coalescing proteins. These transitions involve dedicated proteins that promote coalescence and are facilitated by endo-membranes and cytoskeletal platforms. Moreover, several proteins make use of weak multivalent interactions or conformational templating to drive the formation of large-scale assemblies. Formation of such assemblies is often associated with a change in biochemical activity that can be used by cells to execute biochemical decisions in a localized manner during development and adaption. Since all assembly types impact cell physiology, their localization and dynamics need to be tightly regulated. Interestingly, at least some of the regulatory mechanisms are shared by functional membrane-less organelles and assemblies of terminally aggregated proteins. Furthermore, constituents of functional assemblies can aggregate and become non-functional during aging. Here we present the current knowledge as to how coalescing protein assemblies are spatially organized in cells and we postulate that failures in their spatial confinement might underscore certain aspects of aging and neurodegenerative diseases.

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INTRODUCTION

Many proteins undergo regulated large-scale conformational transitions driving their assembly into multimeric bodies that promote cellular adaptation.¹ In contrast, unwanted structural changes in proteins, caused for example by mutations or quality control defects, can lead to terminal protein aggregation – a hallmark of aging and several degenerative diseases.² Historically the process of protein misfolding has been intensively studied, as it is considered a major threat to cellular physiology. From studies conducted mainly with reporter systems that generate a burden of misfolded proteins we have learned that cells have evolved elaborate quality control mechanisms which can refold or degrade misfolded proteins. Moreover both the artificially induced conditions and different physiological stresses lead to the assembly of protein inclusion bodies. Under physiological heat stress, the coalescence of proteins protects the functionality of protein complexes and does not lead to protein degradation.³ In the case of some proteopathic diseases, the large assembled states have been shown to be less harmful to the organism than oligomeric states.² Perhaps the most extreme example of protein alternative folding are prion diseases such as Creutzfeldt-Jakob's, which are based on the infectious propagation of self-templating alternative protein conformations. However, even for these, which appear to be malignant, there are functional equivalents in biology. For instance prion-like activity appears to have a conserved functional significance in memory encoding.^{4,5} Thus the formation of dedicated deposits represents both physiological and aberrant states respectively. The fate of these coalescing proteins depends on the case involved: some proteins form singular inclusions that are not, for example, shared during cell division, whereas others remain dispersed and can spread during division and between neighboring cells. Thus, elucidating the mechanisms responsible for recognition and routing of proteins into dedicated deposits enables us to understand basic mechanisms of cellular organization and has major implications for biomedical research. To understand how these

different states are discriminated and coordinated during dynamic processes such as cell division, we need a comprehensive investigation of the responsible molecular mechanisms that control protein coalescence in space and time. Here we discuss regulation of the spatial organization of coalescing proteins and provide examples that highlight the consequences of these mechanisms to processes such as aging, memory and degenerative diseases.

Formation of a Single Coalescent Body as a Means of Protecting the Cell and its Siblings

Protein aggregation in cells often results in the formation of a single inclusion body. This phenomenon is conserved from prokaryotic *E. coli* to human cells, indicating that the spatial sequestration of aggregating species into single inclusions is a highly selected trait. For instance aggregate markers^{6,7} such as the small heat-shock protein IbpA⁸ form a single inclusion body in bacterial cells.⁹ Also both fission and budding yeast cells eventually tend to form a single inclusion marked by the disaggregase Hsp104 when experiencing stress-induced protein aggregation.^{10,11} Over-expression of unstable proteins can also lead to the formation of single-compartment specific deposit sites termed IPOD, JUNQ¹² and INQ.¹³ During replicative aging the non-stressed cells, but not their rejuvenated daughter cells, form a single protein deposit.^{14,15} Remarkably the single inclusions from 2 old cells rapidly merge to create a unique deposit after cell-cell fusion through mating.¹⁶ Protein coalescence into a single deposit is not unique to single-celled organisms. After proteasome inhibition, misfolded proteins accumulate at a unique site termed the aggresome.¹⁷ Many proteins accumulate to form the aggresome including Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), huntingtin,¹⁸ α -synuclein,^{19,20} and ataxin-3.²¹

Thus most organisms have cellular mechanisms that spatially constrain protein aggregates to a single deposit (Fig. 1A), which for example enables the asymmetric inheritance of this inclusion during cell division. Indeed,

the fusion of aggregates by the small heat-shock protein Hsp16.^{23,24} Similarly, in budding yeast the singularity of protein inclusions is promoted by the small heat-shock protein Hsp42.^{15,25} The coalescence to a single deposit is conserved from fungi to mammals, as it also promotes the asymmetric inheritance of damaged proteins during mammalian cell division and thereby provides a fitness advantage to cells that are free of damage.²⁶ In spinocerebellar ataxia type-3 aged patients, it was the differentiated but not the stem intestinal cells which accumulated ataxin and polyglutamine aggregates.²⁷ Taken together, a wide variety of examples demonstrates that cells form a single inclusion body which allows for a spatial confinement of protein damage. In the event of cell division, this confinement favors the asymmetric partitioning of the potentially problematic constituents of these structures. Adding to this, the coalescence of proteins to larger deposits is also involved in the asymmetric partitioning of physiologically relevant assemblies.

Many recent studies have highlighted the properties of various assemblies composed of proteins and in many cases RNA to partially isolate themselves from the surrounding cytoplasm through liquid-liquid phase separation.²⁸ Interestingly, these assemblies are reversible and often highly dynamic. The formation of such assemblies governs several cellular responses to environmental alterations.¹ For example, the soluble stress granule associated poly(A)-binding protein Pab1 assembles into hydrogels during thermal and pH stresses, and this phase transition promotes cellular fitness during stress.²⁹ Thus, coalescence of proteins to dedicated deposits is a conserved feature of cells in stressful situations and may also be used during physiological and unstressed conditions.

Protein Inclusions as Encoders of Asymmetrically Inherited Cellular Memories

A conserved process that relies on the mechanisms described above is the recently discovered encoding of memory through the assembly of activated mnemons or

engrams.^{4,5} When exposed to mating pheromone, budding yeast cells grow in a polarized manner (shmoo) toward the source of pheromone. If the mating partner is not encountered then the cell eventually stops shmooing and resumes vegetative growth to produce daughter cells even though pheromone is still present.^{4,30} Remarkably, after resuming a cycling state, cells that have shmooed no longer respond to pheromone; however, daughter cells born from these mother cells still shmoo. A superassembly of the mRNA binding protein Whi3 was identified to encode this pheromone refractory state.⁴ Importantly, this coalesced state of Whi3 remains in the mother cell during cell division, thus allowing the daughter cells to respond to pheromone⁴ (Fig. 1B). Whi3 is the constituting member of mnemons – a class of proteins that are responsible for stable phenotypic states which are only present in those cells that experience the inducing signal. Whi3 aggregation is driven by a Q/N-rich prion-like domain^{4,31} which is commonly found in eukaryotic proteomes,³¹⁻³³ thus giving rise to the question of whether mnemons exist outside of fungi.

In neurons of sea slugs, fruit flies and mice, the mRNA-binding and prion-like domain containing CPEB/Orb2 proteins are required for long-term memory due to their ability to undergo persistent aggregation upon synaptic stimulation.³⁴⁻³⁷ The aggregation of CPEB proteins leads to translation of the mRNA targets, which in turn promote synaptic strength. This implies that mnemon-like activities in proteins are conserved and thus a prevalent form of molecular assemblies in the adaptive arsenal of eukaryotic cells. The overarching hypothesis of CPEB proteins would be that their aggregation at the activated synapse does not spread to neighboring synapses even though CPEB has prion properties in yeast.³⁴ How this coalesced memory-maintenance state is confined to the site of activation from yeast to mammals thus poses a perplexing question still to be answered. Similarly, whether or not the Whi3 mnemon adopts a self-templating prion-like state and how Whi3 super-assemblies are retained in the mother cell are unanswered questions.

How Are Coalescing Assemblies Spatially Confined in Cells?

As already detailed, the coalescence of diffusing particles is a powerful way to generate asymmetry.^{15,23,24} However, this does not explain how coalescent bodies 1) form only in one of the 2 lineages and 2) are inherited by a specific lineage. Work in budding yeast has recently provided interesting insights into the mechanisms responsible for the asymmetric inheritance of different coalescent bodies that seem to use partially overlapping mechanisms. Heat-stress induced protein-coalescent bodies, for instance, localize to the ER³⁸ and preferentially to the vicinity of ER-mitochondria contact sites.³⁹ It has been suggested that these ER-mitochondria interfaces play an important role in asymmetric inheritance because the mitochondrial fission protein Fis1 was required for the maintenance of this asymmetry.³⁹ Surprisingly, it was recently found that at least some of these mitochondrially-targeted aggregated proteins are imported into the mitochondria where they are degraded by proteases such as Pim1.⁴⁰ Interestingly, the actin cytoskeleton also plays a role in asymmetric segregation of protein aggregates. Heat-induced aggregates associate with actin cables, and it has been suggested that this cable attachment results in passive transport of aggregates from the bud to the mother cell due to retrograde flow of polar actin cables.^{41,42} More recently a link between actin, Hsp104 and the vacuolar protein Vac17 has revealed a potential role for endosomes in the collection of aggregates to a vacuole-associated inclusion which promotes their asymmetric inheritance.⁴³ Besides these active mechanisms, it has been suggested that the polarized growth and limited exchange of aggregates between mothers and buds is what largely accounts for the asymmetry.¹⁰ Thus it appears that several overlapping mechanisms ensure the retention of stress-induced inclusions.

In contrast to stressed cells where the aggregates nucleate both in mothers and buds, during replicative aging the coalesced protein deposits appear only in the mother cell. Since the mechanisms ensuring proteostasis are not compromised during early aging,¹⁵ we provided

evidence that inclusions form through the collection of aggregate precursors which accumulate specifically in the mother cell over time. Somewhat similarly to other deposits, age-associated protein aggregates localize to the ER surfaces.¹⁶ Taken together, the consensus from several aggregation models indicates that even though they are membrane-less they localize and make use of endo-membranes to control their assembly and localization (Fig. 1B).

This raises the question: How do the deposits and their precursors interact with cellular membranes? We identified the Hsp40 protein Ydj1 as a membrane anchor for aggregation precursors that were destined for the age-associated deposit.¹⁶ Ydj1 is anchored to ER membranes by being post-translationally farnesylated in its C-terminal CAAX box.⁴⁴ We found that this lipidation of Ydj1 was required for the retention of aggregate seeds in the mother cell¹⁶ (Fig. 1B). This retention required the compartmentalization of the ER membrane by a diffusion barrier at the bud neck region.⁴⁵⁻⁴⁷ The diffusion barriers are comprised of a sphingolipid-enriched lipid domain.⁴⁶ Thus it appears that these diffusion barriers can in part insulate cellular processes by repelling post-translationally lipidated proteins such as those harboring the branched and bulky farnesyl moiety. Since diffusion barriers are also found at the base of many cellular appendages and at the division plane of asymmetrically dividing metazoan cells,⁴⁸⁻⁵⁰ this observation raises many interesting alternatives as to how cells might polarize their components so as to execute compartment-specific tasks. It is noteworthy that diffusion barriers and farnesylated Ydj1 are only confining aggregation precursors but not the large inclusions.¹⁶ It is also interesting to note that, in addition to cytosolic aggregation precursors, misfolded proteins in the ER-lumen are also retained in the mother cell by diffusion barriers.⁴⁶ Taken together, we propose that the ER membrane functions as a platform to disaffiliate proteins which have changed their conformation from their native partners. This involves their recognition and anchorage to the ER by membrane-bound receptors such as DnaJ protein Ydj1, which facilitate the collection of deposit seeds into a single inclusion

body only in the lineage that these precursors have formed.

Interestingly, in asymmetrically dividing neural stem cells of mice the asymmetric partition of ubiquitinated proteins also correlates with the strength of an ER diffusion barrier,⁵⁰ thus suggesting that some aspects are also likely to be conserved between budding yeast and mammalian cells. A recent study identified the deubiquitinase USP19 as an ER-bound receptor for misfolded proteins such as α -synuclein.⁵¹ Intriguingly, this pathway promoted the secretion of misfolded proteins out of the cell, which could be impactful in cell-cell spread of certain aggregates. Thus, there are both similarities, but also clear mechanistic differences to what has been characterized in yeast. In cultured mammalian cells, the aggresome generally co-localizes with the centrosome. Disassembly of the microtubule network leads to dispersed foci¹⁷ without disassembling the aggresome, suggesting that the formation but not the maintenance of aggresome is microtubule-dependent. Of note, a similar mechanism is used by the filamentous fungus *Aspergillus nidulans*, where the coalescence of heat-induced Hsp104-labeled aggregates was promoted by cytoplasmic microtubules and the motor protein dynein.⁵² It might be that an increased concentration of smaller entities around the centrosome is sufficient to nucleate their fusion to a single and larger inclusion. Furthermore the intermediate filament vimentin cages the aggresome¹⁷ and may thus be involved in its maintenance.

In conclusion, many coalescing assemblies are captured by membranous and cytoskeletal structures in cells, which facilitates their spatial control. The most intensively studied budding yeast uses the following mechanisms to segregate protein inclusions to the mother cells: 1) coalesced proteins are collected at single deposit sites; 2) both large inclusions and their precursors are connected to internal membranes; 3) the retention of seeds is achieved through the compartmentalization of endo-membranes by diffusion barriers. However, there are also examples that appear to have escaped the singularity of most inclusions, evidenced most devastatingly by the spread of prion-like diseases.

This gives rise to the question: How do certain coalescing conformers evade the existing confinement mechanisms?

Coalescing Assemblies that Spread Escape the Confinement

Not all aggregating proteins are confined during cell division. A prime example are prions that are formed by self-replicating altered protein conformations.⁵³ Prions were originally identified as infectious proteaceous elements that were later on shown to be composed of amyloid like fold of PrP protein, and to cause neurodegenerative disease.⁵⁴ It has now become evident that a large number of proteins can behave in a prion-like manner, and that this behavior underlies both diverse physiological processes and the pathogenicity of several human neurodegenerative diseases.^{2,55,56} The alternative folds of prions can induce novel phenotypes deriving from a gain in the protein's function or a loss thereof which can in turn be beneficial or detrimental. In addition to canonical prions, proteins that harbor intrinsically disordered domains (IDRs) can adopt alternate non-amyloid-like states of activity which drive heritable traits.⁵⁷ Thus, canonical prions and some IDR-harboring proteins are able to evade the existing confinement mechanisms that were detailed in the previous chapters.

In budding yeast, prions represent heritable phenotypic switches that can drive cellular adaptation to changing environments; and hence prion states, for example the *[PSI+]* and the *[MOT3+]*, are induced by environmental cues.^{58,59,60} The *[PSI+]* prion formed by the altered conformation of the translation-termination factor Sup35 is the best characterized yeast prion. In its prion form, Sup35 exists in the equilibrium of soluble monomers and amyloid oligomers of various sizes. Importantly, the mitotic stability of the prion state critically depends on the existence of small oligomers that can diffuse from the mother to the bud. Oligomers tend to nucleate large inclusions which can be fragmented by the disaggregase Hsp104. If Hsp104 is disabled, propagons are unable to transmit to daughter cells.⁶¹ Similarly,

overexpression of Ydj1, which is involved in the compartmentalization of age-associated aggregates, promotes curing of the [PSI⁺] prion. Thus the transmission of propagons may be partially limited by the same pathway that prevents the diffusion of age-associated aggregate precursors from the aging mothers to the buds. Indeed, we found that detaching Ydj1 from the ER membrane intensified the Sup35 prion phenotype indicative of increased number of spreading propagons.¹⁶ Interestingly, [PSI⁺] can also be propagated in *S. pombe*⁶² and *E. coli*,^{63,64} which in both cases requires the Hsp104/ClpB disaggregase activity for the mitotic spread. Thus, a balance between solubilization and aggregate fragmentation by disaggregases is critical for the mitotic stability of the prion state. However, not all prions depend on fragmentation by Hsp104, some require Hsp70 or Hsp90 chaperones for their propagation.⁵⁷ Overall, it is remarkable that the very same molecules which are induced during stress to prevent protein misfolding are also responsible for the spreading of prions. Furthermore, the fact that prions exist or can spread in organisms ranging from bacteria to fungi to plants to mammals suggests that the mechanisms which seed propagation of conformational states are conserved across most living organisms. Thus the next big challenge is to understand how the conformation-based recognition processes function to control the fate of aggregating proteins.

In mammals the conversion of the soluble prion protein PrP^(cellular) to the prion form PrP^(scrapie) leads to self-sustaining formation of β -sheet-rich assemblies responsible for transmissible spongiform encephalopathies. Similar to yeast prions, PrP^{sc} is found in various forms from small oligomers to large plaques. Mounting evidence suggests that the progression of many incurable neurodegenerative diseases such as frontotemporal dementia, Alzheimer, Huntington's and Parkinson disease might be underscored by prion-like spread (analogous to PrP^{sc}) of aggregating proteins within the nervous system. However, in contrast to the prion PrP^{sc}, the causative misfolded proteins driving these diseases are not considered infectious and are thus referred to as prionoids² – although

the potential human-to-human transmission of proteopathic A β seeds has recently been suggested.⁶⁵ As we have learned from yeast, the key determinant for spreading prions is their ability to self-propagate, which depends on the existence of small diffusible oligomers generated from the fragmentation of larger assemblies. While metazoans lack the Hsp104 disaggregases, the disaggregation is performed by a chaperone complex comprising Hsp110-Hsp40 and Hsp70.⁶⁶⁻⁶⁸ In addition the PDZ serine protease HtrA1 has been found to dissolve and degrade Alzheimer disease-related tau and A β 42 fibrils.^{69,70} Thus it appears that, similar to yeast, the Hsp40 DnaJ proteins are in a key position to regulate the fate of aggregating proteins. In humans the DnaJ-proteins are comprised of approximately 50 proteins and such divergence could provide specificity for a large conformational landscape of different types of aggregates. Moreover, at least some of the human DnaJ-proteins such as DnaJ-A1 harbor a farnesylation motif, which implies that in addition to being adaptors for disaggregation and degradation they might also function as membrane-anchored receptors for aggregates perhaps subjecting them to diffusion barrier-mediated confinement.

Altogether it appears that the mechanisms of confinement and spreading may be dictated by the very same proteins but helped by recognition factors such as DnaJ proteins. Remarkably, the genome of *Plasmodium falciparum* encodes for more than 40 DnaJ proteins.⁷¹ While *P. falciparum* has a small proteome (5268 proteins), nearly a quarter of its proteins contain a prion-like domain, suggesting that the repertoire of Hsp40 may have expanded to cope with such a proteome. This is particularly interesting because this parasite has to adapt to its different hosts' diverse environmental conditions such as temperature that might act as a cue to trigger adaptative prion-like domain containing protein assemblies. Thus, a fascinating avenue of future research is whether the DnaJ proteins and their potential to interact with membranes have co-evolved with the conformational landscape of coalescent proteins.

Are Dysfunctional Phase Transitions and Problems in their Spatial Confinement Drivers of Aging?

Aging is a major risk factor for most neuro-degenerative diseases. However, we know little about how the sporadic forms of these protein-aggregation diseases are triggered and what leads to the progressive and eventually detrimental spread of the aggregation phenotype within the central nervous system. Research on model organisms for cellular and organismal aging has been and will continue to be very informative in understanding the cellular responses to aging and disease-associated aggregation. For instance work on budding yeast has shown that protein aggregates are a contributing factor to yeast-replicative aging – although at present it is not exactly known why age-associated aggregates form and how they limit the replicative life span. Our view is that by harboring a large repertoire of functionally coalescing proteins (e.g. those with prion-like domains), cells may become more susceptible to aberrant protein aggregations if such proteins escape their normal quality control. Such aberrant transitions can contribute to age-associated decline in organismal fitness. Indeed, the Whi3 protein that constitutes the functional memory (mnemon) of pheromone exposure readily aggregates erratically during aging, thereby impairing the mating ability of aged cells.⁷²

Similar to yeast cells, stem cells undergo replicative aging, i.e. their ability to undergo self-renewing divisions is limited.⁷³ Since stem cells are required for tissue regeneration, their declining ability to self-renew may contribute to tissue dysfunction and age-associated diseases. Because stem cells can divide asymmetrically into renewing and differentiating cells there is great interest in attaining an understanding of to what extent accumulation of cellular damages and/or impairment in asymmetric division could potentially contribute to the observed decline of stem cells to undergo self-renewing divisions during aging.⁷⁴ For instance, one possibility is that protein aggregates which may arise during aging could contribute to the observed decline in stem-cell renewal. Importantly, both

diffusion-barrier strength and asymmetric inheritance of damaged proteins decreased with age in the neural stem cells of mice.⁵⁰

In summary, we are beginning to understand how cells organize themselves in response to internal or external changes, either by reducing non-functional and hazardous protein species through the formation of inclusion bodies, or by organizing their biochemical activities through the formation of large-scale assemblies. In both cases the outcome critically depends on how these assemblies are distributed in space. Thus, understanding how distinct assemblies are formed, recognized and controlled in space and time are major questions for future research.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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